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The development of resistance to taxanes is the major clinical limitation in using these agents for treating breast cancer. P-glycoprotein (P-gp) is a multidrug transporter, the product of the *MDR*1 gene and the major known mechanism of resistance to taxanes. In this project we sought to identify non-MDR1 mechanisms of resistance by developing relevant cellular models. We developed models of both uterine sarcoma and breast cancer resistant to taxol alone (mediated by P-gp) and the combination of taxol with PSC 833, a potent inhibitor of P-gp. The uterine sarcoma cells showed changes in β-tubulin isotype profiles whereas the breast cancer cells did not. Both cell types were demonstrated to have alterations in apoptotic proteins, specifically BCL-2 up regulation and BAX down regulation. Genomic profiling yielded numerous genetic differences between the selected variants, the significance of which needs further investigation.

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INTRODUCTION

Paclitaxel (Taxol®), a diterpene plant product isolated from the bark of the Pacific yew tree, *Taxus brevifolia*, has been shown to have significant activity in advanced ovarian and breast malignancies (1-3). Tubulin is the target molecule which mediates the cytotoxicity of paclitaxel (4). The best understood mechanism of resistance to paclitaxel is the multidrug resistant (MDR) phenotype mediated by the multidrug transporter P-glycoprotein (P-gp) (5). Other possible mechanisms of resistance to paclitaxel include alterations in microtubule composition and/or dynamics and changes in regulation of apoptosis. Alterations in α- and β-tubulin subunits have been described both in human and non-human paclitaxel resistant cell lines (6, 7). We have previously examined the contribution of P-gp and tubulin alterations as mechanisms of taxol resistance by performing Luria-Delbruck fluctuation analysis with the human sarcoma cell line MES-SA (8-11). Selection of mammalian cells with paclitaxel has been shown to result both in increased expression of P-gp and in tubulin alterations (11-14). While paclitaxel treatment leads to activation of apoptosis (15), the molecular details of microtubule alterations inducing resistance to paclitaxel remain obscure. Indeed the occurrence of MDR mutants in these selections has complicated the analysis and made characterization of microtubule alterations difficult. In order to identify mechanisms of drug resistance other than MDR, we have isolated drug resistant cell lines by performing selection with incremental increases in paclitaxel concentration in the presence of the MDR modulator PSC 833 (PSC). We hypothesized that the presence of this potent MDR modulator would negate the selective advantage of P-gp expression, such that cells with other mechanisms of drug resistance would survive.

BODY

MATERIALS AND METHODS

A) Cell Lines

MDA-MB-435-S is a human breast carcinoma cell line obtained from ATCC. This line and its variants were maintained in DMEM supplemented with 10% FCS, Penicillin 100 U of penicillin/mL, and 100 µg of streptomycin/mL (all from Irvine Scientific, Santa Ana, CA). Cells were grown in a humidified chamber with 5%CO2. Cells were transfected with the murine ecotropic receptor using G418 selection in preparation for further gene expression experiments. These parental cells were serially selected with increasing concentrations of paclitaxel (5nM, 10nM, 15 nM, 20 nM, 30nM) in the presence and absence of PSC833. Surviving cells were exposed to higher concentrations of drug with resultant surviving variants designated MDA-T20(no PSC833), and MDA-TP20 (selected with PSC833).

As control cell lines, we used the MES-SA cell line, derived in our laboratory from sarcomatous elements of a mixed Mullerian tumor, and its MDR variant MES-SA/DX5, which have been previously described (9, 10). Two variants were selected, one for resistance to paclitaxel alone (MES-SA/T30), the other by co-selection with PSC833 (MES-SA/TP30). These variants were derived by continuous stepwise selection in up to 30nM paclitaxel.

(B) Drugs and Reagents

Paclitaxel (Taxol) was obtained from Bristol-Myers Squibb Company (Wallingford, CT). The cyclosporin analogue SDZ PSC 833 (valspodar) was generously supplied by Novartis Corp. (East Hanover, NJ). Stock solutions of 0.1 mM of these drugs were stored

in absolute alcohol at -20 °C. All other chemicals and pan-β monoclonal antibody were purchased from Sigma chemical Co. (St. Louis, MO).

(C) 3-[H]-Paclitaxel Accumulation studies

MDA-435 cells and variants growing in log phase were incubated in the presence of 3-[H]-paclitaxel for 1 hour (time required to reach equilibration) in presence and absence of PSC-833. Cells were washed, pelleted, and Paclitaxel accumulation analyzed via Cerenkov scintillation counting.

(D) Western blots

Cells were harvested in log phase of growth and pellets were resuspended in lysis buffer containing Tris HCl ph 6.80, MgCl2 1 mM, 2 mM EGTA, and 0.2% Tween 20. Total protein was quantified by the Lowry assay and 100 ug of each sample was prepared in SDS then boiled before being applied to a 12% SDS-polyacrylamide gel. Proteins were then blotted onto a Hybond-ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) using a Sartorius apparatus (Hayward, CA). The membrane was blocked with buffer containing 5% milk and 1% bovine albumin then incubated 2 hours at room temperature with the respective specific antibodies.

(E) Reverse Transciptase Polymerase Chain Reaction (rt-PCR).

Total RNA extraction and rt-PCR were performed as previously described (16). PCR was performed in a Perkin-Elmer Cetus DNA thermal Cycler (Norwalk, CT) using the following profile: 30 sec at 94°C, 1 minute at 55°C (60°C for MRP), and 2 minutes at 72 °C. The amplimers used in this study were synthesized by Operon Technologies (Alameda, CA). Amplimers for mdr1 were 3020-3037 (forward) and 3168-3187 (reverse). Amplimers for MRP were: 3069-3086 (forward) and 2522-2541 (reverse). Ribosomal

cDNA (1846-1826 reverse; 1501-1520 forward) was used as an internal control. We designed the following primers for analysis of the β-tubulin isotypes (Arabic numerals refer to the gene, Roman numerals refer to the tubulin protein isotype class):

-M40 (class I) forward primer: 5′ (-42,-22) CCA TAC ATA CCT TGA GGC GA3′

-M40 reverse primer: 5′ (226, 246) GCC AAA AGG ACC TGA GCG AA 3′

-β9 (class II)forward primer: 5′ (1131,1150) CGC ATC TCC GAG CAG TTC AC 3′

-β9 (Class II) reverse primer: 5′ (1301, 1319) TCG CCCC TCC TCC TCC TCG A 3′

-β4 (Class III) forward primer: 5′ (1,15) ATG AGG GAA ATC GTG 3′

-β4 (Class III)reverse primer: 5′(223,243)AAA GGC CCC TGA GCG GAC ACT 3′

-5β (Class IVa) forward primer: 5′ (-85,-68) TCT CCG CCG CAT CTT CCA 3′

-5β (Class IVa) reverse primer: 5′(167,186) TCT GGG GAC ATA ATT TCC TC 3′

-β2 (Class IVb) forward primer: 5′(-43,-23) GTC TAC TTC CTC CTC TTC CC 3′

-β2 reverse primer: 5′(291,300) GTT GTT CCC AGC ACC ACT CT 3′

These primers were designed using published sequence data for M40, 5β and β2 isotypes or in the case of the β4 isotype a consensus forward primer and partial sequence information generously provided by Kevin Sullivan (Scripps research Institute, La Jolla, CA). Primers for M40, 5β, β2, and β4 were designed to span introns. In the case of class II isotype, a partial nucleotide sequence of the c-terminal region was obtained by screening expressed sequence tags from the EMBL GeneBank (EST T03799), using the peptide sequence previously reported by Cowan et al. (EST T03799) (17).

We have tested each sample over a range of different number of PCR cycles and at different concentrations of cDNA. Ribosomal cDNA was used as an internal control for standardization and comparison of samples. cDNAs were first adjusted in order to provide ribosomal PCR products which differed by less than 10%. PCR samples were analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide, and analyzed by densitometric reading of bands on an Alpha Innotech IS-1000 image analyzer (San Leandro, CA).

(F) Measurement of Cytotoxicity

Approximately 5,000 cells were seeded in 96-well plates and incubated with or without drugs for 72 hours at 37°C in an atmosphere of 5% CO2. Growth inhibition was evaluated by the MTT colorimetric method on quadruplicate assays as previously described (18). The absorbance was quantitated with a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA). The IC_{50} (drug concentration resulting in 50% inhibition of MTT dye formation, compared to controls) was determined directly from semilogarithmic dose-response curves.

RESULTS

A) Selection of paclitaxel resistant MES-SA cells

Our initial studies were performed on the MES-SA uterine sarcoma line due to difficulties with clonally selecting paclitaxel resistant breast cancer lines. After altering tissue culture conditions, a step-wise incremental selection process was utilized to establish resistant breast cancer cells which also contrast to the uterine sarcoma cells with regard to some cellular properties (*MDR1* expression, *P53* status). Continuous, step-wise selection to 30 nM paclitaxel resulted in a cell line (MES-SA/T30) that was 60-fold resistant to the selection conditions by a modified MTT colorimetric assay (18), with no difference in the proliferation rate relative to the wild-type control. This variant was cross-resistant to the MDR substrates doxorubicin (15-fold), vinblastine (16-fold), vincristine (20-fold), colchicine (4-fold) and the taxane analogue docetaxel (Taxotere-, 550-fold). 2 μM PSC completely restored the sensitivity to wild-type levels. Rt-PCR assays confirmed the presence of *MDR*1, and immunoblotting with C219 monoclonal antibody, revealed the presence of its gene product, P-glycoprotein (Figure 1). [^{3}H]-paclitaxel accumulation in these cells found a 4-fold decrease in intracellular retention of drug reversible in the presence of 2 μM PSC (Figure 2). The doxorubicin-selected MES-SA variant, MES-SA/Dx5, was included as a positive control for *MDR*1 expression in these assays.

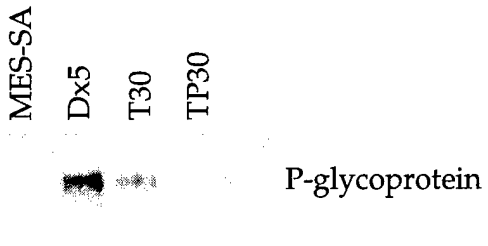

Figure 1.

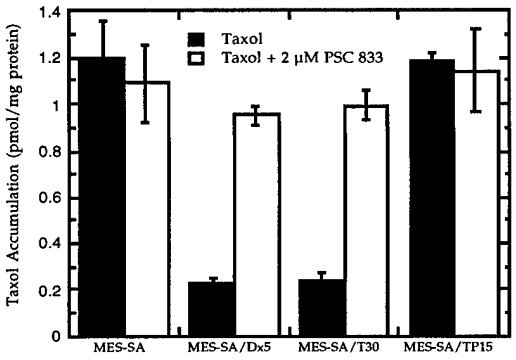

Figure 2.

Figure 1. Expression of P-glycoprotein (P-gp) in MES-SA cells and selected variants. Note the increased expression in DX5 cells and cells selected in paclitaxel (MES-SA-T30) and the lack of expression in the parental line and cells co-selected with PSC-833 (MES-SA-TP30).

Figure 2. The comparative intrcellular accumulation of 3H-paclitaxel in MES-SA cells and selected variants in the presence and absence of PSC-833.

B) MDA-435-S variants- human breast cancer line

MDA-435-S cells were transfected with the murine ecotropic receptor by electroporation and selected in 1mg/ml G418 in preparation for subsequent transfection and designated MDA-ECO. Cells selected with stepwise increasing concentrations of paclitaxel were serially passaged. Initially, cellular passage was complicated by the requirement of a minimal cell density for continued growth. Eventually cells were selected capable of surviving in 20nM paclitaxel (usual IC_{50} about 1-2 nM) and were designated MDA-T20. A parallel selection was performed in the presence of 2uM PSC-833. The resulting cells are designated MDA-TP20. These variants were then further selected in 30 nM paclitaxel in the presence or absence of PSC833 and the surviving cells designated MDA-T30 and MDA-TP30 respectively. The cytotoxicity of various compounds incubated with and without modulators was determined by MTT assay. IC_{50} values were determined directly from semi-logarithmic dose-response curves.

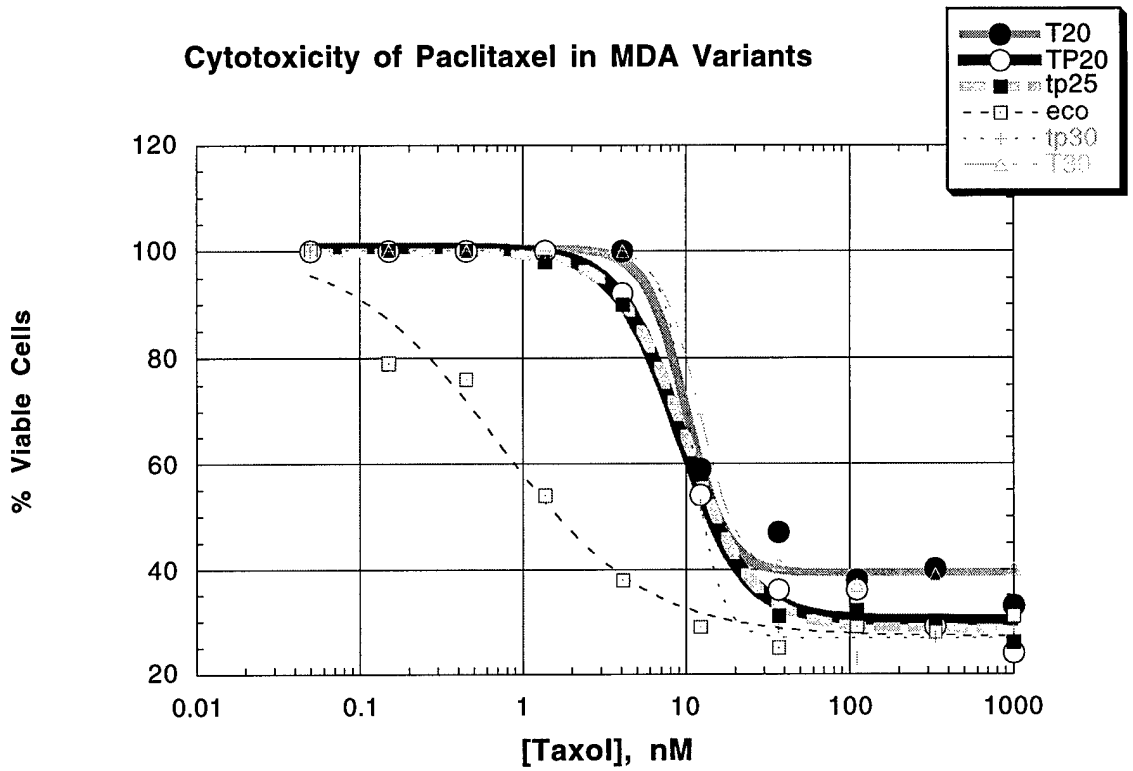

Figure 3. Representative MTT assessing Cytotoxicity of Paclitaxel in MDA-ECO and variants.

Exposure of parental MDA-435-ECO cells to successively increasing concentration of paclitaxel resulted in variants demonstrating relative resistance (Table 1, Figure 3). These cells were maintained in the absence of paclitaxel and maintained their phenotype implying a genetic basis for their phenotype. The cytotoxicity to other chemotherapeutic agents demonstrated cross resistance to the vinca alkaloids in the MDA-T20 variant but not the MDA-TP20 variant. No cross resistance to either doxorubicin (an *MDR1* substrate) or cisplatin (non-*MDR1* substrate) was observed. Analysis of the growth properties of the variant cell lines showed no difference in their

proliferative capacity or growth rate when grown out of drug nor was there any difference in the DNA profile assessed by flow cytometry.

Cell Line	Paclitaxel	VCR	VBL	doxorubicin	Cisplatin
MDA-ECO	1.7±0.6 nM (1.0)	0.1nM	0.45nM	45nM	12uM
MDA-T20	19.4±2.7 nM (11)	0.95nM	22nM	40nM	8nM
MDA-T30	25 nM (15)	N.D.	N.D.	N.D.	N.D.
MDA-TP20	14.2±3.2nM (8)	0.09nM	0.75nM	30nM	5.5.nM
MDA-TP30	18 nM (11)	N.D.	N.D.	N.D.	N.D.

Table 1. Cytotoxicity of cytotoxic agents in MDA variants measured by MTT assay in the absence of PSC-833. Expressed as IC_{50}. The paclitaxel data represents the results of 6 experiments over 8 months. Numbers in parentheses are the fold-resistance seen compared to the parental cells. Abbreviations are VCR-vincristine, VBL vinblastine, N.D.- Not done.

C) Exclusion of P-glycoprotein mediated resistance

As noted above, increased P-gp expression is a major mechanism of acquired resistance to paclitaxel. Since some of the variants showed some cross resistance suggestive of a membrane transporter, we used rt-PCR analysis and radiolabeled paclitaxel accumulation experiments to exclude this mechanism.

1) MES-SA variants

While selection with paclitaxel alone resulted in an *MDR*1 variant (MES-SA/T30), co-selection with PSC resulted in a cell line (MES-SA/TP30) that was negative for *MDR*1 and its gene product (Figures 1). MES-SA/TP30 cells are approximately 15-fold resistant to the taxanes paclitaxel and docetaxel, and the presence of PSC in cytotoxicity assays had no effect on the sensitivity profile of MES-SA/TP30 cells. They accumulate wild-type levels of [^{3}H]-paclitaxel eliminating a transporter-mediated mechanism of resistance (Figure 2).

2) MDA-435 variants

We compared the parental and variant lines with regard to membrane transporters as a mechanism of the observed resistance. MTT cytotoxicity assays in the presence of the P-glycoprotein inhibitor PSC-833 showed no effect in the MDA-TP20cells and slight sensitization in the MDA-T20 cells which was not surprising given the selection conditions. However, assessment of *MDR1* by rt-PCR showed lack of expression at the mRNA level in all three lines. Furthermore, intracellular accumulation assays using [3-H]-paclitaxel showed no difference between the three cell lines in the presence and absence of PSC-833 implying lack of a functional membrane transporter as a resistance mechanism in these cells (Figure 4).

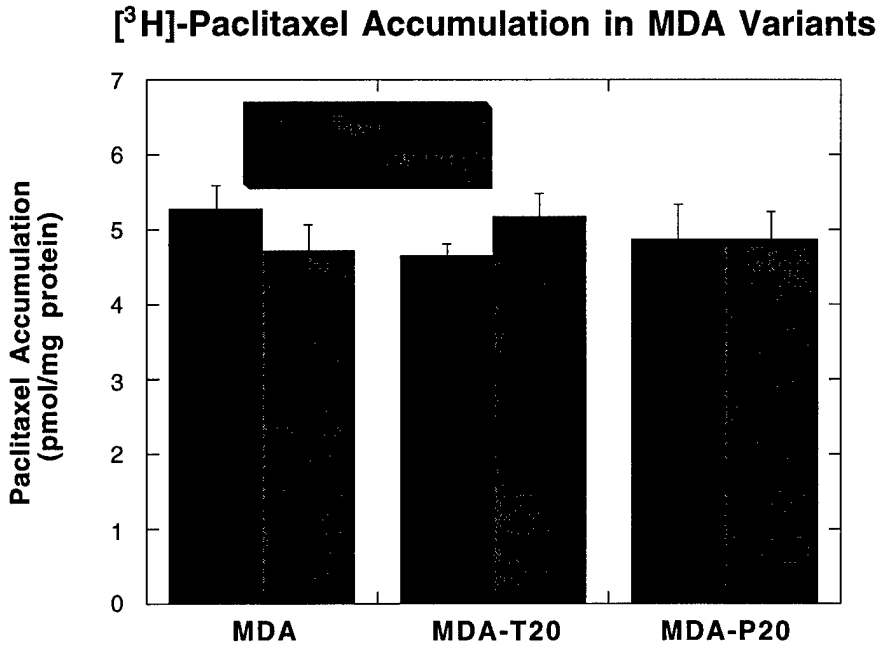

Figure 4. Accumulation of radiolabeled paclitaxel in MDA variants. Parental cells (MDA-ECO, labelled MDA), and variants selected in paclitaxel alone (MDA-T20), or paclitaxel in the presence of PSC-833 (MDA-TP20, labelled MDA-P20 here), were exposed to labelled paclitaxel in the presence (hatched bars) or absence (solid bars) of PSC-833. After washing, the remaining (intracellular) labelled paclitaxel was measured.

D) Tubulins in Paclitaxel-Resistant Variants

i) MES-SA variants

Analysis of the β-tubulin content, the cellular target for paclitaxel, by rt-PCR using isotype-specific amplimers revealed an increase (3- to 4-fold) in the human β9-tubulin (Class II) isoform exclusively in MES-SA/TP30 cells (Figure 5), but not in MES-SA/T30 cells. Immunoblotting for total α- and β-tubulin expression in MES-SA/TP30 cells, however, did not reveal any significant differences in protein levels relative to the wild-type control. (Figure 6).

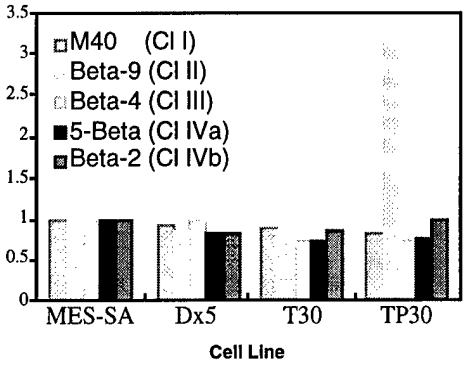

Figure 5.

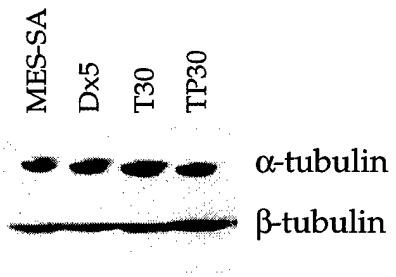

Figure 6.

Figure 5. The β-tubulin isotype profiles for MES-SA cells and selected variants. The results are representative of mRNA levels relative to parental cells. The levels were assessed with isotype specific rt-PCR amplimers (semi-quantitative).

Figure 6. A Western blot of MES-SA cells and variants comparing total α- and β-tubulin protein levels.

1) MDA-435 variants

We examined the parental and resistant lines for total tubulin content using pan-α and pan-β antibodies and saw no differences. Semiquantitative rt-PCR was performed on cellular preparations identically prepared from parental and variant cells in log growth phase. Primer pairs were as previously described for the following genes: β-tubulin isoforms (β1, β-2, M40, β-4, 5-β, β-9), *MDR1*, apoptotic proteins (*BCL-2*, *BAX*), and ribosomal RNA as a control. Samples were analyzed by polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide and quantitated using an Alpha Innotech IS 1000 image analyzer All reactions were normalized to ribosomal RNA. Using previously defined isotype specific primers to assess β-tubulin isotype profiles in these variants we saw differences of uncertain significance (Table 2). Isotypes

5-β, β-4, and β-9 have been reported to be associated with paclitaxel resistance in certain model systems and there are slight alterations noted, but no changes more than 2-fold.

Cell Line	M40	β-1	β-2	β-4	5-β	β-9	*MDR1*
MDA-ECO	1.47	-	2.22	1.75	1.68	2.30	neg
MDA-T20	1.34	-	2.88	2.34	2.25	2.8	neg
MDA-TP20	1.30	-	1.49	1.54	1.67	1.43	neg

Table 2. Relative mRNA levels of the β-tubulin isotypes expressed in MDA variants. Levels are expressed relative to rRNA levels and were analyzed semi-quantitatively as described.

E) Apoptotic Proteins in Paclitaxel Resistant Variants

There have been many recent reports of paclitaxel resistance secondary to failure of regulation of apoptosis, particularly with regard to *BCL-2*, *BAX*, and *BCL-X_L* (19-21). We therefore examined apoptosis in the MES-SA and MDA-435 variants.

i) MES-SA variants

MES-SA/TP30 cells are also cross-resistant to non-MDR DNA alkylating agents, such as cisplatin (3-fold), carboplatin (3-fold) and thiotepa (2-fold) which indicated a potential alteration in apoptotic regulatory proteins. Paclitaxel has previously been found to phosphorylate the apoptotic inhibitors Bcl-2 and the long-form of Bcl-X (Bcl-X_L) in a variety of cell types (19,20), inactivating their normal function in response to cellular damage (21). Further, short-term acute treatment with paclitaxel results in

increased Bcl-2 levels in MCF-7 cells (22). A cell line which is resistant to paclitaxel could potentially become resistant to the selection conditions by increasing Bcl-2 above wild-type levels. In fact, immunoblotting revealed elevated Bcl-2 expression in MES-SA/TP30 cells (Figure 7). Although Bcl-X levels were barely detectable by immunoblotting (data not shown), immunoprecipitation revealed no significant differences in the long-form of Bcl-X (Figure 8).

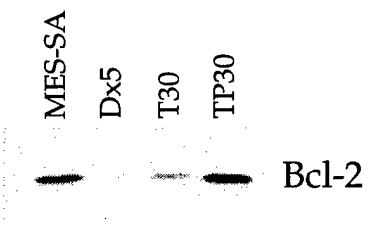

Figure 7. Comparative levels of BCL-2 in MES-SA cells and variants as assessed by immunoblotting. Note the marked increase in the MES-SA-TP30 cells which do not express P-gp.

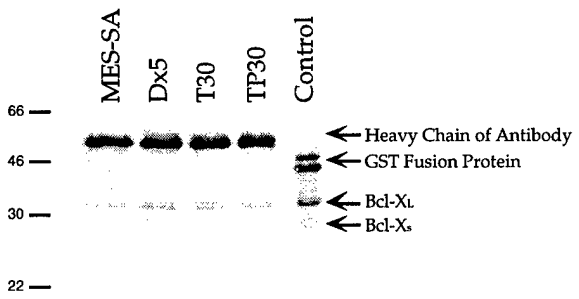

Figure 8. Relative amounts of BCL-X_L in MES-SA cells and variants assessed by immunoprecipitation.

ii) MDA-435-S variants

While the selected MDA-T20 and TP20 cells did not demonstrate cross resitance to cis-platin or thiotepa, we found alterations in apoptotic regulatory proteins. As shown in Table 3 and Figure 9, there are increases in the anti-apoptotic *BCL-2* and decreases in the pro-apoptotic *BAX* messages resulting in 6-7 fold alterations in the *BCL-2 / BAX* ratio between the resistant selected variants and the parental control cells.

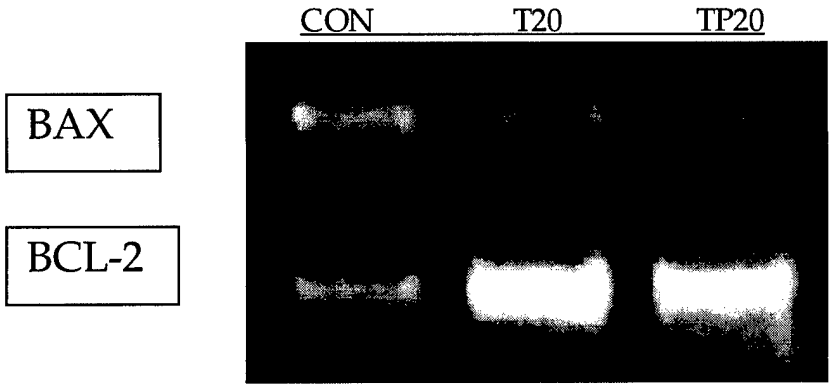

Figure 9. Rt-PCR of parental (CON) MDA-MB-435 cells compared to paclitaxel selected variants (T20 and TP20). These PCR products were electrophoresed and stained with ethidium bromide and analyzed quantitatively with an Alpha Innotech iamge analyzer.

Cell Line	BCL-2	*BAX*	*BCL-2/BAX*
MDA-ECO	1.0	1.0	1.0
MDA-T20	3.67	0.49	7.5
MDA-TP20	2.75	0.4	6.9

Table 3. Relative *BCL-2* and *BAX* in MDA variants assayed by rt-PCR, normalized for ribosomal RNA and relative to MDA-ECO.

Immunoblots on cellular protein verified the *BCL-2* changes in the paclitaxel selected variants, though *BAX* was not detectable with immunoblotting. *BCL-X* immunoblotting performed on cellular immunoprecipitates did not demonstrate differences between the selected variants (data not shown).

DISCUSSION

Resistance to anticancer drugs in breast cancer cells may develop through alterations in drug efflux, modification of the drug target (tubulin), and a downstream failure of apoptotic regulation. By co-selecting tissue culture variants with paclitaxel and the P-gp inhibitor PSC833, we have successfully developed stable cellular models of human breast cancer that demonstrate non-*MDR1* paclitaxel resistance. Our results in these models may be summarized as follows: 1) step-wise selection with paclitaxel yielded cells 10 to 60-fold resistant to paclitaxel, 2) Co-selection with PSC833 suppressed the development of P-gp expressing variants, 3) uterine sarcoma cells resistant to paclitaxel demonstrated hypersensitivity to vinca alkaloids while some breast cancer cells showed cross resistance to vincas, 4) uterine sarcoma paclitaxel resistant variants had an elevation in Class-II β-tubulin, whereas no significant alterations were seen in the β-tubulin isotype profile in breast cancer cells, 5) Alterations in apoptotic regulatory proteins were seen in both uterine sarcoma and breast cancer cells, and 6) Genomic profiling of these models yielded numerous genetic differences between the selected variants though the significance of such differences is unclear.

The alterations in apoptotic proteins at the RNA and protein level are of interest and we plan to exploit these models to better understand the steps leading to this phenotype (e.g. *RAF1* kinase, *BCL-2* phosphorylation). Additionally, the cross resistance to vincas, a class of drugs with activity in breast cancer) displayed in the MDA-T20

variant suggests alterations in tubulin properties (polymerization, binding sites, MAPs) that we are investigating further.

(a) Taxane resistance-membrane transporters.

In this report, we used the human breast cancer cell line MDA-MB-435-S to select variants resistant to paclitaxel and used PSC-833 selection to suppress the emergence of P-gp expressing variants. We demonstrated in a uterine sarcoma line (MES-SA) that selection with paclitaxel in the absence of MDR1 inhibition may result in variants over-expressing P-gp (MES-SA-T30), whereas co-selection with PSC833 results in paclitaxel-resistant cells without P-gp expression (MES-SA-TP30) (23). In the breast cancer model, both variants, selected in the presence or absence of PSC-833, did not express P-gp. A non-MDR1 transporter was excluded by comparative intracellular accumulation of radiolabeled paclitaxel. Another group, using the MDA-MB-435 line showed that overexpression of P-gp could render these cells paclitaxel-resistant (24). Furthermore, p185c-erbB2 also correlated with resistance and both of the effects on *MDR1* and *C-ERBB2* were reversed by treatment with modulators (verapamil) or blocking antibodies, respectively. When the MDA-MB-435 cells were transfected with p185ErbB2 with subsequent overexpression, the activation of p34Cdc2 kinase by paclitaxel was blocked, apparently by association with p21Cip1. Interestingly, our preliminary results with genomic profiling revealed a trend toward decreased *ERBB2* mRNA. The discrepancy may be explained by multiple functions of receptor tyrosine kinases such as ErbB2, post-transcriptional regulation that is not detectable by looking only at the message, or other divergent properties between the MDA-MB-435 cells that may have accumulated in culture conditions.

In our cellular models, the resistance to paclitaxel has been stable for over six months in tissue culture implying a stable genetic mechanism. The differences between our observations in paclitaxel selected breast cancer cells and uterine sarcoma cells may reflect important biological differences based upon cell of origin or may reflect individual properties of cells selected for tissue culture growth. We and others have documented different forms of p53 in the two parental cell lines and this is under further investigation presently. The two lines and selected variants also differ with respect to cross resistance to non-taxane tubulin poisons. This difference provides MDA-TP20 cells as a model for breast cancer cells that are selectively resistant to taxanes and comparison with MDA-T20 cells may help elucidate taxane specific drug resistance mechanisms.

(b) Taxane resistance- tubulin alterations

Paclitaxel resistance has been observed as a result of changes in the target, tubulin. Alterations in total tubulin content (6), β-tubulin isotype composition (12), and β-tubulin sequence (14) all have the potential to give rise to paclitaxel resistance. In the uterine sarcoma cells, MES-SA/TP30, there were significant elevations in the Class II b-tubulin associated with paclitaxel resistance. Further experiments to modulate the Class II isotype may clarify the contribution of this change to the resistance phenotype in the MES-SA cells. In contrast, in the breast cancer cell lines, there were no significant alterations in total tubulin content (α or β) nor β-tubulin isoform profile when assessed by semi-quantitative rt-PCR with isotype specific primers. The experiments reported here do not exclude tubulin mutations such as those reported by Giannakakou, et.al. with resultant alterations in microtubule polymerization properties. Other possibilities

include changes in microtubule associated proteins such as the increased sensitivity to paclitaxel seen in p53 mutant cells and associated with elevated MAP4 expression (26). This change in MAP4 results in hypersensitivity to vincas which is the phenotype we observe in sarcoma cells (MES-SA/TP30), however in the breast cancer models, there is actually cross-resistance to the vincas. To date we have been unable to distinguish a cellular property related to tubulins that explains the resistance profiles observed or the cross resistance to vinca alkaloids in the MDA-T20 cells.

(c) Taxane resistance- Apoptotic proteins

Numerous cellular events may result in activation of the apoptotic apparatus. Microtubule disruption with subsequent failure of mitosis or dysregulation of the dynamic instability of microtubules may serve as such events. Blagosklonny, et.al. have shown in one model system a pathway whereby paclitaxel treatment leads to elevations in RAF1 kinase with subsequent phosphorylation of BCL-2 (inactivation) and activation of apoptosis (27,28) In the breast cancer cell line MCF-7, Huang, et al. note that estrogen increases BCL-2/BAX ratios with a resultant inhibition of paclitaxel induced apoptosis (29). In breast cancer cells selected for doxorubicin resistance (MCF/Adr), bcl-2 levels were down-regulated approximately 10-fold, while bax and bcl-xl levels were unchanged. Though in this model there was apparently a contribution of P-gp (30). In both the sarcoma and breast models, we observed alterations in BCL-2 to BAX ratios which were stable even after the selection pressure of drug was removed. The stability of the phenotype in the absence of paclitaxel makes it unlikely that the cellular alterations are induced by drug treatment. The alterations in BCL-2 and BAX mRNA and protein expression do not fully explain the observed resistance profile (e.g. why

there is not cross resistance to cisplatin), nor does it account for the cross resistance observed in MDA-T20 cells. We plan to perform bcl-2 antisense experiments with regulatable vectors to clarify the role of bcl-2 in taxane resistance in these cells.

The relationship between p53 status, bcl-2, bax, and bcl-x and drug induced apoptosis is also complex. In ovarian carcinoma cells selected for resistance to cisplatin there was no change in bcl-2 or its homologues. Resistant cells showed inactive p53. In both parental and selected cells apoptosis after drug treatment was associated with elevated levels of Bak and Bax after drug-induced damage and that functional p53 is required for the effect with cisplatin but not with paclitaxel. This is consistent with our findings here that altered levels of Bcl-2 and bax in the context of mutant p53 could lead to paclitaxel resistance but not cisplatin resistance (31).

Finally, the relationship between membrane transporters (P-gp, MRP, etc.) and the apoptotic machinery is complex and apparently not separate and may be confounded by the p53 status of the cells. Strobel, et al. have described a model where elevated BAX expression enhances taxol-induced apoptosis by affecting an unknown membrane efflux pump (32, 33).

REFERENCES

1) Wall,M.E. and Wani, M.C. Camptothecin and taxol: discovery to clinic-thirteenth Bruce F. Cain Memorial Award Lecture, Cancer Res. 55:753-60, 1995.

2) Gelmon, K.A. Biweekly paclitaxel (Taxol) and cisplatin in breast and ovarian cancer, Semin Oncol. 21:24-8, 1994.

3) Caldas, C. and McGuire, WP. Taxol in epithelial ovarian cancer, Monograph Natl Cancer Inst. 155-9, 1993.

4) Parness, J. and Horwitz, S.B. Taxol binds to polymerized tubulin in vitro, J. Cell Biol. 91:479-87, 1981.

5) Horwitz, S.B., Cohen, D.,Rao, S., Ringel, I. Shen H.J., and Yang, CP. Taxol: mechanisms of action and resistance, Monogr Natl Cancer Inst 55-61, 1993.

6) Schibler, M.J. and Cabral, F. Taxol-dependent mutants of Chinese hamster ovary cells with alterations in α- and β-tubulin, J Cell Biol. 102:1522-31, 1986.

7) Ohta S., Nishio, K., Kubota, N., Ohmori, T., Funayama, Y., Ohira, T., Nakajima, H., Adachi, M., and Saijo, N. Characterization of a taxol-resistant human small-cell lung cancer cell line, Japn J Cancer Res. 85:290-7, 1994.

8) Luria, S.E. and Delbruck, M. Mutation of bacteria from virus sensitivity to virus resitance, Genetics. 28:491-9, 1943.

9) Harker, W.G., MacKintosh, F.R., and Sikic, B.I. Development and characterization of a human sarcoma cell line MES-SA, Cancer Res. 3:4943-50, 1983.

10) Harker, W.G. and Sikic, B.I. Multidrug (pleiotropic) resistance in doxorubicin-selected variants of the human sarcoma cell line MES-SA, Cancer Research. 45:4091-6, 1985.

11) Beketic-Oreskovic, L, Duran, GE, Chen, G, Dumontet, C, and Sikic, BI. Decreased mutation rate for doxorubicin resistance and suppression of *MDR*1 gene activation by PSC833. *Journal of the National Cancer Institute* 87: 1593-1602, 1995.

12) Haber, M, Burkart, CA, Regl, DL, Madafiglio, J, Norris, MD, and Horwitz, SB. Altered Expression of Mβ2, the class II β-tubulin isotype, in a murine J774.2 cell line with a high level of taxol resistance. *Journal of Biologial Chemistry* 270(52): 31269-31275, 1995.

13) Burkhart, CA, Kavallaris, M, and Horwitz, SB. Inducible expression of the class II β-tubulin isotype in murine fibroblast cells. *Proceedings of the American Association for Cancer Research* 39: 1295, 1998.

14) Giannakakou, P, Sackett, DL, Kang, Y-K, Zhan, Z, Buters, JTM, Fojo, T, and Poruchynsky, MS. Paclitaxel-resistant human ovarian cancer cells have mutant β-tubulins that exhibit impaired paclitaxel-driven polymerization. *Journal of Biologial Chemistry* 272(27): 17118-17125, 1997.

15) Bhalla K, Ibrado A.M., Tourkina E., Tang, C., Mahoney M.E., Huang Y. Leukemia 7:563-8, 1993.

16) Chen, G., Jaffrezou, JP., Fleming, W.H., Duran, G.E., and Sikic, B.I. Prevalence of multidrug resistance related to activation of the mdr-1 gene in human sarcoma mutants derived by single-step doxorubicin selection, Cancer Res. 54:4980-7, 1994.

17) Cowan, N.J., Lewis, S.A., Sarkar, S., and Gu, W. Functional versatility of mammalian β -tubulin isotypes. In: R.Maccioniu and J. Arechaga (eds.), The Cytoskeleton in Cell Differentiation and Development, pp.157-66:ICSU Press, 1986.

18) Twentyman, P.R., Fox, N.E., and Rees, J.K. Chemosensitivity testing of fresh leukaemia cells using the MTT colorimetric assay, Br J Haematol. 71:19-24, 1989.

19) Haldar, S, Basu, A, and Croce, CM. Bcl-2 is the guardian of microtubule integrity. *Cancer Research* 57: 229-233, 1997.

20) Pruchynsky, MS, Wang, EE, Rudin, CM, Blagosklonny, MV, and Fojo, T. Bcl-X_L is phosphorylated in malignant cells following microtubule disruption. *Cancer Research* 58, 3331-3338, 1998.

21) Haldar, S, Jena, N. and Croce, CM. Inactivation of Bcl-2 by phosphorylation. *Proceedings of the National Academy of Science USA* 92: 4507-4511, 1995.

22) Huang, Y, Ibrado, AM, Reed, JC, Bullock, G, Ray, S, Tang, C, Bhalla, K. Co-expression of several molecular mechanisms of multidrug resistance and their significance for paclitaxel cytotoxicity in human AML HL-60 cells. *Leukemia* **11(2):** 253-257, 1997.

23) Duran GE, Mallarino MC, Dumontet C, Sopchak L, Wang Y, and Sikic BI. Paclitaxel resistant mutants with altered expression of Class II beta-tubulin and apoptotic regulatory proteins. Proc AACR, 39:190, 1998.

24) Yu D, Liu B, Jing T, Sun D, Price JE, Singletary SE, Ibrahim N, Hortobagyi GN, Hung MC. Overexpression of both p185c-erbB2 and p170mdr-1 renders breast cancer cells highly resistant to taxol. Oncogene 16:2087-94 (1998).

25) Yu D, Jing T, Liu B, Yao J, Tan M, McDonnell TJ, Hung MC. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol Cell 2:581-91 (1998).

26) Zhang CC, Yang JM, White E, Murphy M, Levine A, Hait, WN. The role of MAP4 expression in the sensitivity to paclitaxel and resistance to vinca alkaloids in p53 mutant cells. Oncogene 16:1617-24, 1998.

27) Blagsklonny MV, Schulte T, Nguyen P, Trepel J, Neckers LM. Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involves c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. Cancer Res 56:1851-4, 1996.

28) Blagsklonny MV, Giannakakou P, el-Diery WS, Kingston DG, Higgs PI, Neckers L, and Fojo T. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. Cancer Res 57:130-5, 1997.

29) Huang Y, Ray S, Reed JC, Ibrado AM, Tang c, Nawabi A, Bhalla K. Estrogen increases intracellular p26bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. Breast Cancer Res Treat 42:73-81 (1997).

30) Ogretman, B, Safa, AR. Down-regulation of apoptosis-related bcl-2 but not bcl-xL or bax proteins in multidrug resistant MCF-7/Adr human breast cancer cells. Int J Cancer 67:608-14, 1996.

31) Jones, NA, Turner J, McIlwrath AJ, Brown, R, and Dive C. Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and trhe relationship between bax and bak up-regulation and the functional status of p53. Mol Pharmacol 53:819-26, 1998.

32) Strobel T, Swanson L, Korsenmeyer S, Cannistra SA. BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway. Proc Natl Acad Sci 93:14094-9, 1996.

33) Strobel, T, Kraeft, S-K, Chen LB, and Cannistra SA. BAX Expression Is Associated with Enhanced Intracellular Accumulation of Paclitaxel: A Novel Role for BAX during Chemotherapy-induced Cell Death. Cancer Res. 58:4776-4781, 1998.

Bibliography of Publications Supported by This Grant

Jaffrezou J-P, Dumontet C, Derry WB, Duran G, Chen G, Tsuchiya E, Wilson L, Jordan MA, and Sikic BI: Novel mechanism of resistance to paclitaxel (Taxol) in human K562 leukemia cells by combined selection with PSC 833. *Oncology Res.* 7: 517-527, 1995.

Dumontet C, Duran GE, Steger KA, Beketic-Oreskovic L, and Sikic BI: Resistance mechanisms in human sarcoma mutants derived by single-step exposure to paclitaxel (Taxol). *Cancer Res.* 56: 1091-1097, 1996.

Dumontet C, Duran GE, Steger KA, Murphy G, Sussman H, and Sikic BI: Differential expression of tubulin isotypes during the cell cycle. *Cell Motility Cytoskel.*, 35: 49-58, 1996.

Dumontet C and Sikic BI: Mechanisms of action of and resistance to anti-tubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol* 17: 1061-1070, 1999.

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Mallarino MC, Duran GE, Dumontet CM and Sikic BI. Mechanisms of resistance in a human sarcoma cell line continuously selected with paclitaxel (Taxol®, TAX) and SDZ PSC 833 (Abstract #1906), Proceedings of the American Association for Cancer Research. Vol 36: 320, 1995.

Dumontet CM, Duran GE and Sikic BI. Mechanisms of resistance to paclitaxel (Taxol®, TAX) in human sarcoma mutants derived by single-step selection (Abstract #1907), Proceedings of the American Association for Cancer Research. Vol 36: 320, 1995.

Mallarino MC, Duran GE, Dumontet C, Cluck M, Mangili A and Sikic BI. The spectrum of resistance to paclitaxel in human sarcoma and breast cancer cells selected with and without the multidrug resistance (MDR) modulator PSC 833 (Abstract #2987), Proceedings of the American Association for Cancer Research. Vol 37: 437, 1996.

Duran GE, Mallarino MC, Dumontet C, Sopchak L, Wang Y and Sikic BI (Abstract #1299), Paclitaxel resistant mutants with altered expression of class II beta-tubulin and apoptotic regulatory proteins (Abstract #1299), Proceedings of the American Association for Cancer Research. Vol 39: 190, 1998.

Keer HN, Duran GE, Wang YC and Sikic BI. Non-*MDR1* models of resistance to paclitaxel in MDA 435 cells (Abstract #2673), Proceedings of the American Association for Cancer Research. Vol 40: 404, 1999.